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Microbiome Changes during Tuberculosis and Antituberculous Therapy

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SUMMARY

The critical role of commensal microbiota in the human body has been increasingly recognized, and our understanding of its implications in human health and disease has expanded rapidly. The lower respiratory tract contains diverse communities of microbes known as lung microbiota, which are present in healthy individuals and in individuals with respiratory diseases. The dysbiosis of the airway microbiota in pulmonary tuberculosis (TB) may play a role in the pathophysiological processes associated with TB disease. Recent studies of the lung microbiome have pointed out changes in lung microbial communities associated with TB and other lung diseases and have also begun to elucidate the profound effects that antituberculous drug therapy can have on the human lung microbiome composition. In this review, the potential role of the human microbiome in TB pathogenesis and the changes in the human microbiome with *Mycobacterium tuberculosis* infection and TB therapy are presented and discussed.

INTRODUCTION

Tuberculosis (TB) affects around 9.4 million people and kills more than 1.3 million per year in the world (1, 2). TB has always been considered a major health concern and especially for low-income countries. Despite a cure rate of 85 to 95% for TB with treatment, depending on the population studied, up to 12% of patients still die (3) and up to 7% relapse (4). Treatment failure cannot be fully explained by low patient compliance or drug resistance. Coinfection or a history of a prior infection with organisms like *Pseudomonas* or a disorder in lung microbiota might be involved in the pathogenesis of TB and may influence the treatment outcome (5). Thus, characterizing the whole human microbiome during TB infection and therapy might be critical to understanding the progress, persistence, and recurrence of this disease. The Human Microbiome Project (2008 to 2012) was an initiative sponsored by the U.S. National Institutes of Health (NIH) (6) aiming to understand healthy human microbiota at different body sites. Since then, there have been efforts to characterize the composition of the lung microbiota and their association with TB (5, 7–9). TB may be the result of complex microbial community interactions rather than a single causative agent as traditionally considered. Furthermore, *Mycobacterium tuberculosis* infection requires long-term combined antibiotic treatment, which can alter the lung microbial community profile and subsequently affect the outcome of the treatment (Fig. 1). Here we discuss changes of the microbiome associated with TB, TB therapy, and host risk factors for the disease.

THE HUMAN MICROBIOME IN TB

The human body is a habitat of simultaneously occurring complex communities of microorganisms where site-specific, unique bacterial populations exist under different selection pressures (10). The human body contains diverse microbial communities in niches like the gut (11), oral cavity (12), vagina (13), skin (14), and also the lower respiratory tract, a site that was previously considered "sterile" (15). The term "microbiota" refers to the microbes that live in a specific location, while the term "human microbiome" refers to the collective genomes of the complete microbiota present in the human body and the surrounding environmental conditions (16, 17). Microbial dynamics and balance in their composition and abundance in health can be disrupted, resulting in dysbiosis and the proliferation of pathobionts, organisms implicated in pathological immune response and disease (18). These changes in microbial profile may be a result of host factors such as

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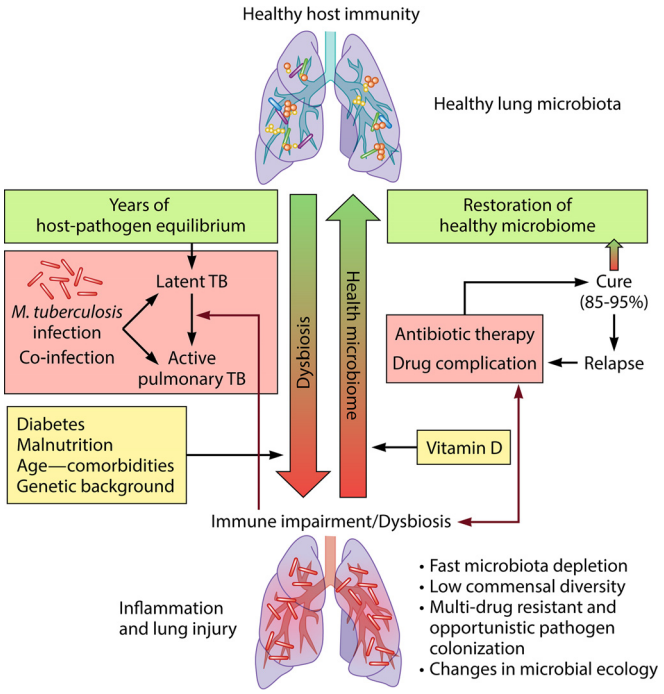

FIG 1 Transition of a healthy balance between host immunity and microbiota into dysbiosis resulted from TB infection and TB therapy. In the healthy state (top), host immunity and microbiota interact with each other to maintain a balance. When TB infection occurs, infection is contained in a latent TB state or progresses to active TB (red box on the left). In dysbiosis (bottom), inflammation develops, and colonization resistance to pathogens is weakened. The genetic make-up of the host and comorbidities like diabetes and malnutrition can influence disease progression and dysbiosis. Antibiotic treatment for TB accelerates dysbiosis by changing the landscape of microbial ecology, which could also affect the resolution of TB infection (red box on the right). The restoration of a healthy host-associated microbiome and the immune system should be explored further in regards for the prevention and treatment of TB (green box on the right).

inflammatory responses and availability of host nutrients for microbes. The physiological activity of commensal microbiota and its metabolites may have beneficial effects and may play critical roles in human physiology, including metabolism, anti-inflammatory activity, shaping of the immune system, homeostasis, and production of vitamins and energy sources (19, 20). Studies of microbial colonization of the intestine have also shown the importance of the microbiome in stimulating and fostering the development and maintenance of innate and adaptive immune responses that help maintain normal homeostasis (21–28).

While significant changes in the respiratory and intestinal microbiota after *M. tuberculosis* infection have been reported (5, 7–9, 29, 30), there is still no clear consensus on how microbiome diversity differs quantitatively in *M. tuberculosis*-infected individuals versus healthy controls or whether specific changes in microbiota status may dictate the prognosis of patients with *M. tuberculosis* infection. Dickson and colleagues have proposed a model for the cycle of dysbiosis, i.e., the alteration in the composition of the microbiome (31), in respiratory inflammation that may apply to *M. tuberculosis* infection. In this model, dysbiosis in the respiratory tract leads to immune response dysregulation in the host, which subsequently alters the environment in favor of the growth of certain microbes. This becomes a vicious cycle that promotes further dysbiosis and inflammation (32). Dysbiosis of the airway microbiota of pulmonary TB patients may contribute to the pathophysiological processes associated with TB disease; i.e., susceptibility, progression, and chronicity of lung disease (33). The term dysbiosis is rather ambiguous, given the amount of individual variability observed in different microbial profiles (34). One aspect that can be measured though is microbial diversity, i.e., the richness and abundance of species within (alpha) or between (beta) samples (34). Microbial diversity appears to be important for the control of inflammatory responses in the lung, especially during early childhood (35). Decreased intestinal microbial diversity and alterations in its composition have been reported with aging (36), as well as with several TB-associated comorbidities like diabetes (37–39) and malnourishment (40, 41). It is uncertain whether the observed microbiome changes could increase the risk of developing active TB in diabetic patients (42) or of reactivating latent TB infection in the elderly (43). The role of gut microbiome immaturity observed in malnourished individuals is even more difficult to dissect, as the relationship between TB and malnutrition is bidirectional, i.e., active TB leads to weight loss, and being underweight constitutes a known risk factor for TB (44). Furthermore, malnutrition is present in other conditions that can lead to an immunodeficient state and may increase the risk of tuberculosis (45, 46).

Despite the differences between lung and gut mucosal biology (47), there is evidence of a complex balance between mucosal immunity and TB. In the TB mouse model, aerosol *M. tuberculosis* infection has shown significant gut microbiota changes in mice (29). A study of a cohort of humans has reported the presence of *Helicobacter pylori* to be negatively associated with progression to active TB. In that study, patients with latent *M. tuberculosis* infection that progressed to develop active TB were about 50% less likely to be *H. pylori* seropositive than the patients whose *M. tuberculosis* infection remained latent (48). A protective role of the gut microbiota in lungs may be achieved via enhancement of primary alveolar macrophage function, as has been demonstrated in the case of *Streptococcus pneumoniae* infection (49).

All this evidence points out a regulatory influence of the gastrointestinal (GI) microbiome on lung immunity, the so-called "gut-lung axis" (50) (Fig. 2). This explains why commensal flora depletion in the gut is related to the severity of inflammatory response in the lung, at least in experimental mice (51). In line with this observation, streptomycin-associated expansion of *Bacteroidetes* in the intestinal microbiota exacerbates the severity of experimental hypersensitivity pneumonitis in a mouse perinatal model (52). Both the lung and intestinal microbiota should be taken into consideration in the pathogenesis, treatment, and future prevention of TB.

LUNG MICROBIOME CHANGES DURING TB

TB is caused by the *M. tuberculosis* complex and can affect the lungs (pulmonary TB) as well as other sites (extrapulmonary TB). Once *M. tuberculosis* reaches the lung alveoli, the pathogen can either immediately multiply and progress to acute active pulmonary TB or, most commonly, persist in latency within macrophages for years until later reactivation upon altered host immune conditions that can be either physiological (such as aging) or externally acquired (HIV coinfection or use of immunosuppressants such as anti-tumor necrosis factor alpha [anti-TNF-α] agent) (53, 54). Although there have been reports of coinfections with differ-

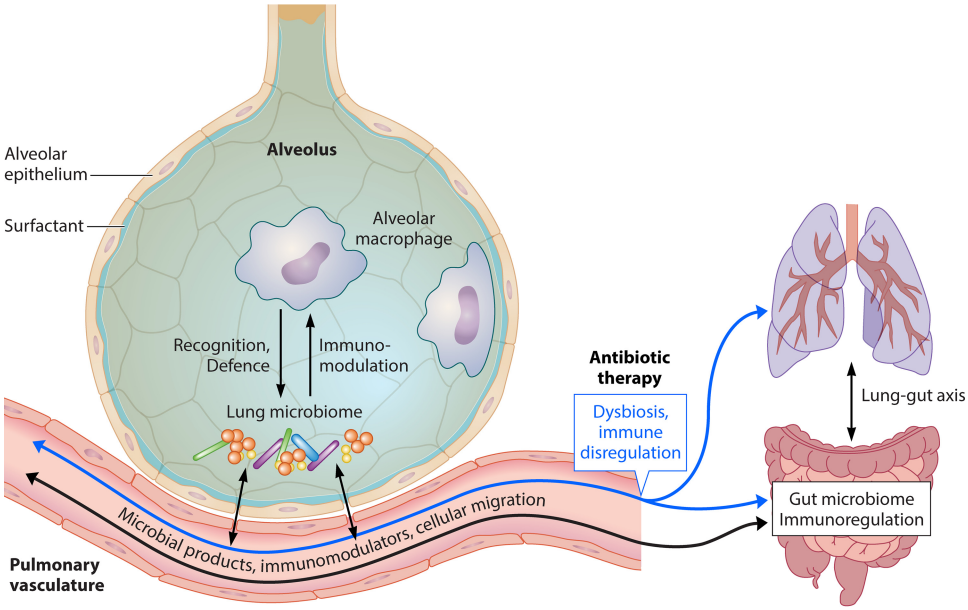

FIG 2 The intestinal microbiome can interact with the lung microbiome via the so-called "gut-lung axis" (50). The intestinal microbiome, through microbial products and immunomodulators released upon recognition of commensals and pathogens by intestinal immune cells, can regulate lung immunity and influence the lung microbiome. Commensal flora depletion, and dysbiosis caused by antibiotic therapy in the gut, can lead to an unbalanced inflammatory response in the lung (51).

ent *M. tuberculosis* strains (55) or even different *Mycobacterium* species (56) in active pulmonary TB, the human lung microbiome associated with TB is still largely undefined. As the alterations in microbial communities have been linked to the state of health of the host shifting into disease-associated states in localized diseases (57, 58), as well as systemic disorders (59), it is critical to understand TB with respect to the surrounding microbial communities. Dynamics of microbial proliferation and elimination from a body site might be concomitant but not necessarily related to systemic illnesses. To date, few studies have explored the lung microbiome composition in TB patients (Table 1). The sample sizes in these studies have been relatively small, and in most of these studies, a comparison was made between sputum microbiota from TB patients and respiratory secretions or oropharyngeal samples from healthy controls (5, 7, 9, 60). This poses limitations in the interpretation of the results, as it is now known that although the microbial composition in the lung overlaps greatly with the microbial composition in the oral cavity but at lower concentrations, the overall community composition is different (61). From these studies, only one study compared sputum samples from TB patients to sputum samples from healthy controls (30), and another study compared the TB sputum microbiome with that of *M. tuberculosis* culture-negative coughing individuals (8). The study by Cheung and colleagues discovered that *Proteobacteria* and *Bacteroidetes* were predominant in sputum samples from TB patients, while *Firmicutes* was the main phylum in sputum samples from the control group (8). In contrast, Krishna et al. showed *Firmicutes* and *Actinobacteria* to be significantly higher in TB samples, while *Bacteroides* and *Proteobacteria* were higher in healthy controls (30). Cui and colleagues (9) found that bacterial communities from pulmonary TB patients were more diverse and that these bacterial communities exhibited the presence of abnormal genera such as *Stenotrophomonas*, *Cupriavidus*, *Pseudomonas*, *Thermus*, *Sphingomonas*, *Methylobacterium*, *Diaphorobacter*, *Comamonas*, and *Mobilicoccus* (Table 1). The most-recent study by Krishna and colleagues reported the presence of opportunistic bacteria in sputum samples from TB patients before treatment (30). This suggests that the lung microenvironment in TB might become more susceptible to colonization by foreign microorganisms (9). The study by Botero et al., performed on a small number of TB patients ($n = 6$) and controls ($n = 6$), is interesting as it compares microbial communities from the nasal cavity and the oropharynx in both groups (7). This study showed several important results. Their analysis showed how communities for each specific niche cluster together, independent of *M. tuberculosis* infection. Also, they showed that the bacterial and fungal communities were similar in the oropharynx and sputum samples and that they were clearly different from those in the nasal niche. Last, it was found that there were also differences in the oropharyngeal microbiome composition in TB patients and controls (7). A study focused on microbiome characterization from bronchoalveolar lavage (BAL) fluid samples from the lesion-forming lung area and also the healthy or non-lesion-forming side (60) found that the microbiota of intra- versus extra-TB lesions were similar, with *Cupriavidus* as the dominant genus in TB patients. Also, there was an increase in the abundance of *Mycobacterium* and *Porphyromonas* inside TB lesions (60). Here the comparison was made with oropharyngeal samples from healthy controls, which limits its interpretation. This same study showed that the diversity indices for the lesion side were higher than those of healthy controls (60). Diversity indices being higher in TB cases than in healthy controls has been previously reported (9), but again this study compared true sputum from patients versus a mixture of saliva and pharyngeal secretions obtained by deep coughing in the morning. The opposite figure, i.e., diversity indices being higher in healthy controls than in TB cases prior to antituberculous therapy has been

TABLE 1 Summary of lung microbiome changes in TB

Study	Yr	Study location	Type of sample (no. of samples) from:		High-throughput sequencing platform	16S rRNA hypervariable region(s)	Avg no. of sequences per sample	Major finding(s)	Taxa abundant in:	
			TB patients	Control subjects					TB patients	Control subjects
Cui et al. (9)	2012	Shanghai, China	Sputum ($n = 31$)	Respiratory secretion ($n = 24$)	Roche/454	V3	1,307	Sputum microbiota from TB patients were more complex compared to healthy controls. Many foreign bacteria were uniquely found in pulmonary TB patients.	*Phenylobacterium*, *Stenotrophomonas*, *Cupriavidus*, *Pseudomonas*, *Sphingomonas*, *Methylobacterium*, *Diaphorobacter*, *Comamonas*, and *Mobilicoccus*	*Neisseria*, *Porphyromonas*, TM7, *Parvimonas*, *Campylobacter*, *Haemophilus*, and *Fusobacterium*
Cheung et al. (8)	2013	Hong Kong, China	Sputum ($n = 22$)	Sputum[a] ($n = 14$)	Roche/454	V1-V2	23,163	*M. tuberculosis* represented only a very small relative abundance despite the fact that it is considered a causative agent. Diversity of microbiota in TB and healthy control was similar. Six common genera in TB samples were found.	*Mogibacterium*, *Moryella*, and *Oribacterium*	Unclassified *Lactobacillales*
Wu et al. (5)	2013	Shanghai, China	Sputum ($n = 75$)	Throat swab ($n = 20$)	Roche/454	V1-V2	1,710	*Pseudomonas* showed high abundance in recurrent TB patients and treatment failure patients. Reduced frequency and abundance of genera normally found in healthy controls might be associated with the onset and recurrence of TB and treatment failure.	*Streptococcus*, *Gramulicatella*, and *Pseudomonas*	*Prevotella*
Botero et al. (7)	2014	Colombia	Nasal, oropharyngeal, and sputum ($n = 6$)	Nasal and oropharyngeal ($n = 6$)	Roche/454	V1-V2	49,083 (fungal 22,395)	Oropharyngeal microbiota showed differences between TB patients and healthy controls. Oropharyngeal samples resembled sputum microbiota, which were distinct from the nasal communities.	*Streptococcaceae* (oropharyngeal samples), *Candida* and *Aspergillus* (sputum and oropharyngeal)	*Actinomyces*, *Fusobacterium*, *Leptotrichia*, *Prevotella*, *Streptococcus*, and *Veillonella*
Zhou et al. (60)	2015	Nanjing, China	Bronchoalveolar lavage fluid ($n = 32$)	Saliva, pharyngeal secretions ($n = 24$)	Roche/454	V3	3,081	*Cupriavidus* was the dominant genus in TB patients, which differed from healthy controls. *Mycobacterium* and *Porphyromonas* were significantly increased in TB lesions.	*Cupriavidus*, *Mycobacterium*, and *Porphyromonas*	
Krishna et al. (30)	2016	India	Sputum ($n = 25$)	Sputum ($n = 16$)	Ion Torrent	V6-V7	34,144	The relative abundance of *Firmicutes* and *Actinobacteria* was significantly higher in TB samples, and *Neisseria* and *Veillonella* were the two dominant genera after *Streptococcus*.	*Streptococcus*, *Neisseria*, and *Veillonella*	*Streptococcus*, *Gammaproteobacteria*, *Neisseria*, and *Haemophilus*

[a] Sputum samples from individuals with TB-resembling coughing symptoms, but the samples were culture negative for *M. tuberculosis*.

shown by two other groups (5, 30), with cases of recurrent TB appearing to have the least diversity (5). Cheung et al. found diversity to be similar between TB and control samples. In this study, sputum samples from TB patients was not compared to sputum samples from healthy controls but to samples from individuals who had coughing symptoms resembling TB but had *M. tuberculosis* culture-negative results (8).

The initial source of the changes in airway microbiota is still unclear; however, it is presumed that changes occurring in gut microbiota can influence the lung microbiota (62). In the normal healthy state, the host must constantly discriminate between symbionts and pathobionts to organize an adequate adaptive response (63), although it is possible that a strong clearance of *M. tuberculosis* could produce local inflammatory lesions that could increase the possibility of foreign bacteria colonization in the lung. Similarly, *M. tuberculosis* and other less pathogenic mycobacteria such as *M. avium* complex can coexist in some patients as combined mycobacterial infection, particularly in immunosuppressed subjects. Thus, the roles of other microorganisms, including bacteria, fungi, and viruses, as well as their interaction with the host immune system in the progression of TB disease or reactivation of TB infection, may be more important than previously thought. Therefore, structural and functional recovery of normal lung bacterial communities in the lung should be considered in the prevention and treatment of TB (9). Interestingly, exploration of the potential role of the lung microbiome in the response of TB patients to treatment and risk of recurrence has only very recently started to be explored. The lung microbiome has also been evaluated in the context of TB treatment. Wu and colleagues analyzed the sputum microbiota associated with *M. tuberculosis* infection in newly infected, recurrent, and treatment failure TB cases. Genera such as *Bulleidia* and *Atopobium* were found to be abundant in recurrent TB patients compared to new cases. Furthermore, *Pseudomonas* was the predominant genus in the treatment failure group compared to cured new patients, and the *Pseudomonas*-to-*Mycobacterium* ratio was higher in treatment failure cases than in newly infected cases (5). A very interesting finding from two studies is the higher presence of *Prevotella* in healthy controls (5, 7). Inconsistencies found in the studies so far can also be explained by differences in the microbiota across geographical locations (64). A meta-analysis of the lung microbiome in tuberculosis would help determine to which extent geographical stratification can account for these differences.

Finally, the microbiome is not a static entity (65), and there is no consensus on the composition of a "healthy" or "normal lung microbiome" (66). Whether consistent changes in the lung microbiome or a decrease in the composition of bacteria from a specific genus occurs in TB patients are issues that will need to be examined through larger longitudinal studies.

LIMITATIONS AND OBSTACLES IN THE STUDY OF THE LUNG MICROBIOME IN TB

A number of variables can affect the accuracy of the evaluation and correct representation of the lung microbiome composition and relative abundance, and these can impact any of the steps from initial sampling to analysis and results (see Fig. 3 for an overview of the steps involved in assessing the composition of the lung microbiome). The initial sampling step is one of the most important factors influencing assessment of the lung microbiome. While this topic has been extensively addressed in a recent review (66), it is important to highlight the relevance of sampling to available studies of the TB microbiome. For some body sites, different available sampling methods tend to yield similar results (e.g., swab, punch biopsy, and scraping to assess the skin microbiome [14]), or the sample of choice is relatively standardized for the specific body site (e.g., feces for study of the human gut microbiome). However, the same is not true for study of the lung microbiome. The fact that the lung harbors a microbiome is clear, yet how distinct that community is from the oral microbiome remains uncertain and may depend on where in the respiratory tract sampling occurs (61). Indeed, a concern of sampling of the lung microbiome is contamination from the oral microbiome, as access to the lung must necessarily pass through at least a part of the oropharynx. Sampling techniques for the lung microbiome include techniques that avoid oral contamination but are highly impractical (direct tissue sampling), techniques that provide protection against oral contamination but that require invasive access (e.g., BAL) (61), and techniques that carry low risk to the subject but a higher risk of oral contamination (i.e., induced sputum) (67). Unfortunately, these techniques vary widely, with at least one study reporting that BAL and sputum, the most accessible methods, differ greatly in terms of the diversity of the microbiome they detect (68). Most of the studies referred to in this review were done using relatively small numbers of TB patients and controls, and most of them compare sputum samples from TB patients versus oropharyngeal samples in the controls (Table 1 and Fig. 3A). While no existing study of the TB lung microbiome has evaluated direct tissue sampling, one (60) has assessed BAL fluid samples, while five (5, 7–9, 30) have assessed sputum samples. Hence, overrepresentation and/or underrepresentation of particular species in TB patients or controls may be due to the origin of the samples to be sequenced and analyzed. Furthermore, how DNA is obtained from samples (Fig. 3B) has a significant influence on the resulting community at the end of analysis, and different extraction methods can vary widely in organisms they favor or disfavor (69, 70). Existing TB microbiome studies utilized different extraction techniques (e.g., MoBio PowerSoil kit in reference 7 and UltraPure Genome DNA kit in reference 9), and this may underlie some differences between these studies. Also, a minor change in any step from sample collection to extraction to library prep will greatly affect the detection and relative abundance of community members.

Another factor that can influence the lung microbiota profile is the sequencing platform (Fig. 4C). Most of the studies presented in this review were performed utilizing 454 pyrosequencing, which has now been replaced by more-cost-effective platforms. Several other sequencing platforms have emerged in the market that differ not only in the sequencing technology used but also in their read length, throughput per run, and error rates (71). Many newer technologies, such as Illumina platforms, offer improved coverage and depth of sequencing, enabling many more reads per sample. Future studies utilizing such technologies would capture more of the microbial community and perhaps provide increased insight into the complex relationship between TB, the microbiome, and the host. Adding further complexity to this is the choice of the hypervariable region of the 16S rRNA gene to be sequenced. Different regions grant different abilities to differentiate microbial organisms (72, 73), and even comparing adjacent regions (e.g., V4 and V4-5) can produce significant differences in community com-

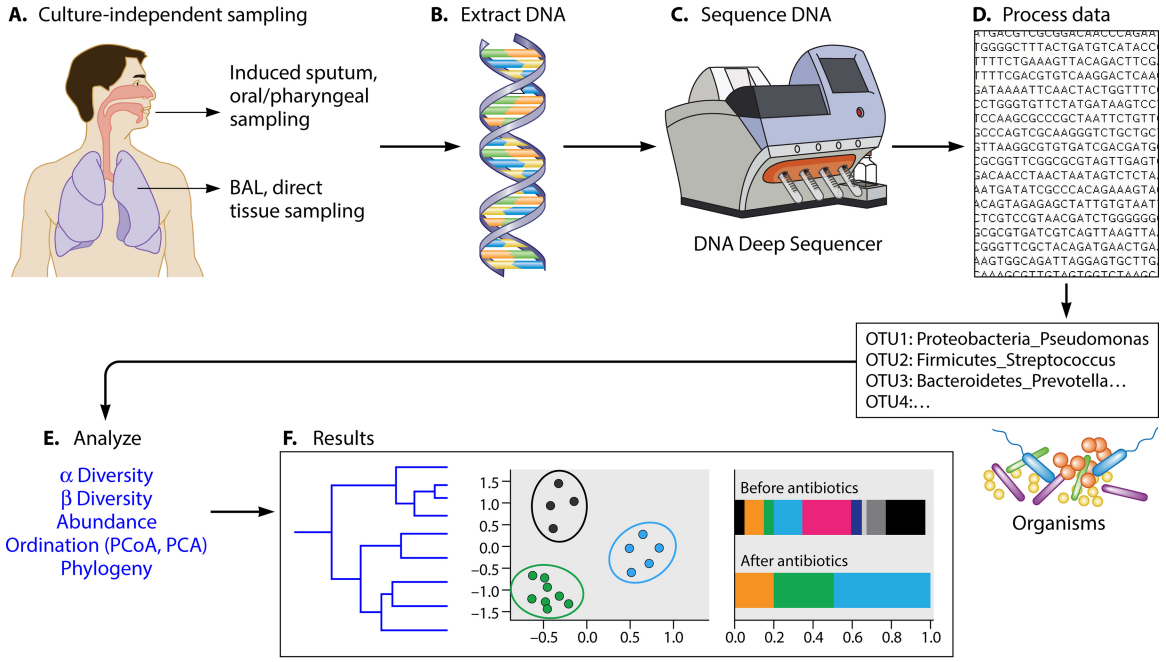

FIG 3 Lung microbiome: from sample to results. To study the lung microbiome, researchers must complete a series of steps. First, samples must be obtained (A) from the subject. This is done in a culture-independent manner. Many methods exist, with induced sputum and bronchoalveolar lavage (BAL) fluid sampling being the most commonly used. In rare cases (e.g., lung transplantation), direct sampling of lung tissue may occur. Following sampling, DNA must be extracted (B) from the sample. Many methods exist by which to isolate DNA, and no one standard method exists. The output will contain DNA from both the host and the microbiome. Once extracted, DNA is then sequenced (C), using next-generation sequencing (NGS) technology. Prior to sequencing, specific genes, such as the 16S rRNA gene for bacteria and the ITS gene for fungi, may be amplified by PCR. Alternatives increasingly utilized include metagenomics and metatranscriptomics, both of which evaluate the entire genomic or transcriptional output of the microbiome. NGS technology produces many gigabases of output, and this output is next processed (D) into information on which microorganisms, i.e., operational taxonomic unit (OTUs), were present in the original sample. That information can then be analyzed (E) using a variety of special bioinformatics algorithms. These methods produce many different kinds of results (F), from tables of organisms to plots of samples clustered by similarity.

position (74). Differences in bacterial profiles obtained from different hypervariable regions of the 16S rRNA gene may reflect differences in the rate of evolution of these regions. No single region is good enough to differentiate all bacteria, but the selection of multiple differing regions (V1-2, V3, and V6-7) makes comparisons between studies challenging, and further studies that evaluate the relative advantage of each region are needed (73). Furthermore, the lack of studies utilizing certain commonly used variable regions (such as V2 alone and V4) (75) means that comparisons with the broader microbiome literature may be complicated by variable region biases. New research approaches that are greatly advanced by recent sequencing technology have also opened the door for more-complex studies that move beyond 16S rRNA sequencing. Metagenomics, or evaluation of all the genomes of all the organisms in a community, is now commonplace, and many groups are expanding their studies to metatranscriptomics (all of the actively transcribed genes in a community) and metabolomics (all of the metabolites being produced by a community), with the latter requiring assays beyond sequencing (76). In one pioneering study of inflammatory bowel diseases, metagenomic analysis revealed that while community composition did not yield great insight, microbial function (as assessed by genes present) was dramatically affected by disease state (77). Similar studies are sorely needed in TB and could greatly advance our understanding of how *M. tuberculosis* interacts with other microorganisms in the host lung. For example, if a future metabolomic study revealed that TB is associated with production of key metabolites by other bacteria, clinicians could target those bacteria and potentially make the host less welcoming to *M. tuberculosis*, easing disease clearance.

Finally, it is important to remember that the microbiome is not solely made up of bacteria. The mycobiome (fungal microbiome) can be identified via sequencing of the internal transcribed spacer (ITS). However, current fungal studies face challenges with multiple species names and with poorly annotated databases that have produced so-called "dark taxa," or database entries that contain a reference sequence but little or no formal taxonomic naming (78, 79). Only one study (7) has examined fungi in the TB microbiome, despite growing evidence that fungal populations can influence the host immune system (80) and, perhaps, the host response to TB. An even more remote frontier is the host virome, or the viruses that dwell among the bacteria and fungi of our micro- and mycobiomes. Very little is known about how the host and its virome interact, and even less is known about this interaction in the lungs (81). The study of the virome is complex, with no complete databases of viral sequences available (82), but growing evidence that viruses can exercise control over other "kingdoms" of life require that future studies of the TB microbiome consider viruses (81). Such studies could uncover novel viruses that might be harnessed to combat *M. tuberculosis* itself or boost helpful microorganisms that could outcompete *M. tuberculosis*.

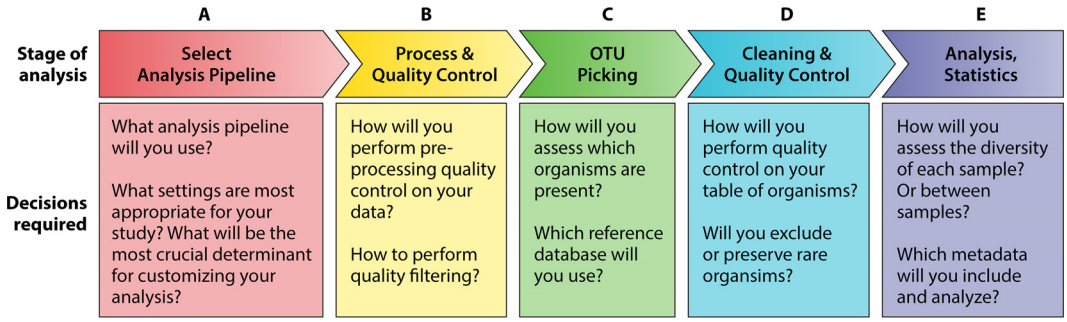

FIG 4 Stages of microbiome analysis and the decisions required at each stage. Analyzing microbiome data involves a series of five key stages, and each stage requires the investigator to answer important questions about how they will perform the analysis. The answers to these questions may be different for individual investigators and studies, and choices made can make comparing data between studies difficult or impossible. First, the researcher must choose which analysis pipeline (A) to use for their analysis. Many different choices exist, and some pipelines incorporate part or all of other pipelines inside of them. This is also the stage where the researcher must consider if they want to change the default settings for their chosen pipeline. Once a pipeline is selected, the first step in analysis is to perform preprocessing and quality control (B). This stage can involve filtering out low-quality data and (for 454 pyrosequencing data) a process known as "denoising", i.e., checking for a special kind of erroneous DNA sequence known as a chimera, or an error that results from a PCR error, can be done at this stage. Next, cleaned data must be turned into information on which organisms are present, a process known as operational taxonomic unit (OTU) picking or clustering (C). An OTU represents an organism or a group of organisms. For example, a researcher might analyze microbiome data at the level of bacterial species, and they might find that their data contains two OTUs representing *Escherichia coli* and *Pseudomonas aeruginosa*. Different reference databases of microorganisms exist, and choosing which to use is an important decision at this stage. Following OTU picking, the data may be further cleaned and processed (D). A researcher might, for example, choose to exclude any organisms or groups of organisms for which there is only one read or a low number of reads, assuming that they are not real. Finally, the last stage involves analyses and statistics (E) of the processed data using one or more of a host of available metrics, statistics, and comparisons. This stage would also involve the inclusion of metadata in the analyses, such as information on drug treatment or disease state of the subject.

TAXONOMIC IDENTIFICATION

Analyzing microbiome data requires multiple steps, and the choices made at each step can impact those downstream. These choices, and some of the key decisions made at each stage, are outlined in Fig. 4. It is important to consider that the evaluating microbiome studies or comparing multiple studies targeting diseases can depend as much on decisions made during data processing steps (83–88).

Pipeline selection (Fig. 4A), the first step in data processing and analysis, is one step which has relevance when assessing the existing pool of studies that have evaluated the microbiome in TB. Of these studies, two (9, 60) utilized custom analysis pipelines, and four (5, 7, 8, 30) made use of QIIME (89), rendering direct comparisons between the results of all of these studies challenging. The reader who is aware of the impact of pipeline selection could choose to compare those studies utilizing QIIME or some other useful software tool such as MOTHUR when planning a research study or potential experimental intervention, reasoning that they may be similar enough to draw some conclusions from. Denoising, a special processing step required to reduce errors introduced by 454 pyrosequencing technology and part of the second step in microbiome analysis (Fig. 4B), is another potential source of differences that should be considered. There is good evidence that small changes to denoising procedures can significantly impact downstream results (84), and most existing TB microbiome studies utilized 454 pyrosequencing (5, 7–9, 60).

One of the most important steps in microbiome data processing is clustering (or picking) operational taxonomic units (OTUs) (Fig. 4C). One way of assigning taxonomic classification is by picking OTUs, and an OTU represents an organism or group of organisms determined from sequence data. Many methods exist to perform OTU clustering, and several make use of reference databases that contain lists of organisms and their corresponding 16S rRNA sequences (74, 90). Comparisons between different reference databases (90) and even between versions of the same reference database (74) have reported divergent results.

Unfortunately, this concern impacts the five available studies of the TB microbiome. Three utilize the Greengenes database (5, 7, 8), and three do not specifically name the reference database (9, 30, 60). An accurate and representative comparison of these studies would likely require a meta-analysis utilizing the same reference database used for all study data sets, but the reader who is aware of these database differences can assess these studies knowing that differences between, for example, Cheung et al. (8) and Wu et al. (5) may arise as much from database choices as from underlying sample differences. Available studies have each made valid choices at steps along the pathway, and it behooves the reader evaluating the literature to keep these choices in mind when comparing studies.

CHANGES IN THE MICROBIOME WITH ANTIBIOTIC AND ANTITUBERCULOUS DRUG THERAPY

Antibiotic therapy has a pronounced effect on human microbiota, with a rapid decrease in diversity, particularly after broad-spectrum antibiotic therapy (91). The impact of antibiotics on the microbiome varies, depending on the antibiotic spectrum, dosage, length of treatment, route of administration, and pharmacological properties of the agent (92). Although the change in the microbiome with antibiotic therapy may be a reversible event, depending on the antibiotic regimen, the recovery time may vary (93). It has been reported that even exposure to a short course of antibiotics can lead to new bacterial populations that become stable for years in the human gut (94). The processes of microbial communities reversing to their initial state are often incomplete (95). Changes in microbial ecology can confer a risk of losing colonization of beneficial microorganisms that are resistant to opportunistic pathogens, leading to new colonization of drug-resistant species and disruption of immune-mediated colonization re-

sistance. Furthermore, commensal flora depletion may also be related to the severity of the inflammatory response in the face of an infection challenge. This hypothesis is supported by a mouse model where antibiotic pretreatment increases *Escherichia coli* pneumonia-induced mortality, presumably by enhancement of Toll-like receptor 4 (TLR4) response (51).

The effect of antituberculous drug therapy on microbiome diversity has only recently been explored. Standard first-line antituberculous therapy offers a unique combination of factors favoring the possibility of a profound change in the microbiome by multiple antibiotic combinations and prolonged use. According to the World Health Organization (WHO), standard first-line TB treatment is based on a combination of four drugs, namely, rifampin, isoniazid, ethambutol, and pyrazinamide for 2 months, followed by rifampin and isoniazid for at least 4 months (1). The intensity and duration of antibiotic therapy exposure are special complications of this condition, as very few other infectious diseases require such a long period of treatment. The majority of commonly used antituberculous drugs are specific to the *Mycobacterium* species in their mechanism of action, with the exception of rifamycins which act through inhibition of the bacterial DNA-dependent RNA polymerase, possess a high intracellular penetration, and constitute very potent drugs for a range of intracellular pathogens (96). The only available study comparing sputum microbiome composition in newly developed TB, recurrent TB, and treatment failure TB patients versus healthy controls (5) does not analyze the effect of antituberculous therapy on the lung microbiome. This study does not report whether sputum samples from the new TB cases were collected prior to antibiotic therapy; instead, it focuses on the analysis of sputum microbiome in TB patients with various disease states. Furthermore, the TB patient group analyzed in this study contain a mixture of monoresistance to rifampin or isoniazid and multidrug-resistant TB (5). A well-controlled study to evaluate the effect of combined TB treatment in the lung microbiome is very much needed.

Among the few reports of the effect of combined antituberculous treatment on intestinal microbiota, Dubourg and colleagues have shown that within a single patient with drug-resistant TB, there is a reduction in the bacterial and fungal diversity with communities dominated by just a few phylotypes with partial colonization by yeast (93). The patient in that report, however, was being treated with multiple second-line broad-spectrum antibiotics, a situation that differs from the usual first-line TB treatment. A potential effect of antituberculous drug therapy that has not yet been fully explored is the role of the reduction or elimination of *Mycobacterium* subspecies that are part of the normal commensal population of the intestine. An example is *M. avium* subsp. *paratuberculosis*, which may be involved in the pathogenesis of Crohn's disease (97–99). If this were the case, antituberculous treatment containing drugs effective against *M. avium* subsp. *paratuberculosis*, such as ethambutol and some rifamycins, may help in the remission of this condition. However, very few studies have reported the effectiveness of antimycobacterial therapy in the remission of Crohn's disease, and they have showed inconsistent results (100, 101).

Rifamycins have *in vitro* activity against *M. tuberculosis*, *M. bovis*, and other atypical mycobacteria, including *M. kansasii*, and a more variable activity against *M. avium* complex organisms. Additionally, they have activity against a broad spectrum of Gram-positive bacteria of the normal skin and respiratory microbial communities, as well as diverse Gram-negative pathogens, including anaerobes from the intestinal microbiome. In fact, a nonabsorbable rifamycin, rifaximin, is currently used for the prevention of recurrent episodes of hepatic encephalopathy because of its ability to reduce enteric ammonia-producing species, for the prevention of traveler diarrhea, as an adjuvant treatment for *Clostridium difficile* colitis, and more recently, for the treatment of colonic dysbiosis present in irritable bowel syndrome (102, 103). The beneficial effect of rifaximin in reducing gut inflammation appears to be due to an increase in *Lactobacillus* abundance and reduction of segmented filamentous bacteria (104).

There are several potential complications associated with antituberculous therapy, hepatotoxicity being the most frequent and feared one (105). Despite its well-known association with antibiotic usage (106), especially when multiple antibiotics are used (107), *Clostridium difficile* diarrhea is a very uncommon event during antituberculous therapy. This may reflect a less pronounced effect of first-line antituberculous drugs in intestinal microbiota compared with other antibacterial agents, or perhaps, it may be related to the bactericidal effect of rifampin *per se* against this pathogen (108).

CONCLUSIONS

It is important for clinicians and investigators to further explore the link between changes that occur in the lung microbiome and tuberculosis, as these may constitute a risk factor for tuberculosis development and failure of treatment. Comorbidities and antibiotic therapy impose changes in the human microbiome and could affect the course of the disease and treatment outcomes. Further studies to elucidate the role of the microbiome and essential nutrients could lead to newer and promising treatment options for TB. All of these critical issues may have an impact in the control and transmission of the disease. Structural and functional restoration of the normal bacterial communities may have an impact in the prevention and treatment of tuberculosis.

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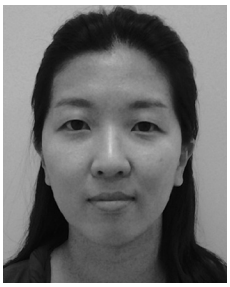

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